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# Determination of Aflatoxins Using High-Performance Liquid Chromatography with Optimized Fluorescence Detection

**Jong-Gyu Kim**†

*Department of Public Health, College of Natural Sciences, Keimyung University, Taegu 704-701, Korea*

# HPLC에 의한 aflatoxin 분석법에 관한 연구—형광 검출의 최적 조건

김종규†

*계명대학교 자연과학대학 공중보건학과*

**ABSTRACT**—A postcolumn derivatization method was tried for the simultaneous determination of four major aflatoxins ($B_1$, $B_2$, $G_1$, and $G_2$) by high-performance liquid chromatography with fluorescence detection. As compared with a previous precolumn derivatization method, it was found that the postcolumn derivatization combined with an electrochemical cell (Kobra cell) was less time-consuming, safer, improved the sensitivity and selectivity, and provided good recoveries for aflatoxin $B_1$ (88.9%) and $G_1$ (100.5%). This method showed linearity from 10~100 ppb for aflatoxin $B_1$ and $G_1$, and from 3~30 ppb for aflatoxin $B_2$ and $G_2$. However, aflatoxin $B_2$ and $G_2$ were not detected satisfactorily although they showed good resolution.

**Key words** □ Aflatoxins, HPLC, fluorescence detection, postcolumn derivatization

Aflatoxins are a group of toxins produced by some *Aspergillus flavus* or *A. parasiticus* Link molds.[1] The toxins are of serious concern because these compounds are among the most potent carcinogens known. Aflatoxins are also directly toxic at relatively low concentrations, 0.5~10 mg/kg of body weight for most animals.[2]

A variety of techniques have been used to separate and identify the four major naturally occurring aflatoxins, namely $B_1$, $B_2$, $G_1$, and $G_2$. Quantitative methods available for the determination of aflatoxins involve chemical and biological procedures.[3-7] Thin-layer chromatography (TLC) is still used widely for the estimation of aflatoxins but high-performance liquid chromatography (HPLC) is often used where the advantages of increased sample throughput with unattended operation are desired. If accurate quantification is required, the TLC method requires the use of a densitometer, which is an expensive piece of equipment, so HPLC becomes an economically viable alternative.

Much early work on aflatoxin separation was done on normal phase columns following the methods of Pons,[8] but more recent methods have tended towards reverse phase separations.[9-11] Several investigators have used reverse phase HPLC which provides more reproducible separations because the solvent systems are not so affected by environmental changes. Another advantage of reverse phase HPLC is the economy and safety of water compared with organic solvents for the mobile phase. Also most of the researchers coupled fluorescence detection with reverse phase HPLC. In this work, the simultaneous determination of four aflatoxins were studied in order to extend the scope of analysis by HPLC with fluorescence detection and to improve the sensitivity and selectivity of the method.

## MATERIALS AND METHODS

### Apparatus and analytical conditions

The HPLC equipment was comprised of Nova-pak $C_{18}$ column (15 cm × 3.9 mm I.D.), an M510 pump, an M746 integrator, and an M470 fluorescence detector (excitation at 360 nm, emission at 440 nm, set from the results of wavelength scanning) (Waters, Milford, MA, U.S.A.). Sample injection was done on a Rheodyne injector (Rheodyne) with

† Author to whom correspondence should be addressed.

a 20 μl sample loop. The mobile phase was water-methanol (58+42, v/v) with the addition of 119 mg of potassium bromide and 100 μl of nitric acid (65%) per liter. The chromatograms were obtained at ambient temperature with the mobile phase at a flow-rate of 1.0 ml/min.

### Chemicals

All reagents used were of analytical grade purity or better. Aflatoxin standards for HPLC injection were purchased from Supelco (Bellefonte, PA, U.S.A.) The standard solutions were diluted prior to analysis.

### Media and microorganism

Yeast-extract sucrose (YES) broth medium was used for aflatoxin production. A volume of 1 *l* of this liquid medium contains 20 g of yeast-extract and 200 g of sucrose. The aflatoxin-producing mold was *Aspergillus parasiticus* ATCC 15517.

### Sample

The spore suspension of *A. parasiticus* ATCC 15517 was inoculated in the YES broth, and incubated at 28°C for 7 days. This was expected to produce the four aflatoxins, and this cultured medium was used as the samples for the analysis of aflatoxins.

### Extraction of aflatoxins and clean-up

For the extraction of aflatoxins, 5 m*l* of sample and 400 mg of sodium chloride were placed in a blender jar. A volume of 25 m*l* of 60% methanol solution was added and the mixture was blended at high speed for 1 minute. The extract was diluted with the same amount of distilled water and filtered through filter paper (Whatman No. 4).

The extracts were purified by an immunoaffinity column, Aflaprep® (Rhône-Poulenc Diagnostics Ltd. Glasgow, Scotland). This clean-up column, which is a 1 m*l* syringe-barrel column that contains immobilized monoclonal antibodies to aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$, is filled with a preserved buffer solution. The procedure used for clean-up was identical to that reported in detail previously by Kim.[12] In brief, 10 m*l* of filtrate was transfered into the glass syringe barrel and pushed through the affinity column with slow steady pressure at the flow rate of ca 1 drops/sec by using the pump unit. Two times the column was washed by passing 10 m*l* of distilled through it. A 1.0 m*l* of HPLC grade methanol was passed through the column, and the eluate containing the aflatoxins was collected in a glass vial. The methanol eluate was used for HPLC detection.

### Derivatization

For the precolumn derivatization, the eluate was evaporated to dryness under a stream of nitrogen gas, and trifluoroacetic acid (TFA) was added before redissolving the residue in an appropriate volume of injection solvent. TFA treated standards and sample extracts were injected on the HPLC column.

For the postcolumn derivatization, a Kobra Cell, an electrochemical cell, was fitted between the HPLC column and the detector. This automatically generated the derivatization agent, bromine, from potassium bromide present in the mobile phase. Standards and the untreated methanol eluate were injected in the HPLC column, then in the Kobra cell for the postcolumn derivatization.

## RESULTS AND DISCUSSION

First a mixture of four aflatoxin standards was directly injected onto the column without any form of derivatization to enhance detectability. Only two aflatoxins were separated from the mixture in about 20 minutes as shown in Fig. 1(1). Because aflatoxin $B_1$ and $G_1$ do not fluoresce as much as $B_2$ and $G_2$, they were not detected in the analytical condition.

After derivatizing with TFA, the results in Fig. 1(2) were obtained with fluorescence detection. The four aflatoxins were fully separated and readily detected. However, the figure establishes that aflatoxin $B_2$ and $G_2$ were unaffected by the derivatization process.

Fig. 1 demonstrates how derivatizing helps separate aflatoxins from an interference and improve detector sensitivity for aflatoxin $B_1$ and $G_1$, now $B_{2a}$ and $G_{2a}$ due to the chemical reaction. The sensitivity of the two aflatoxins actually improved due to the precolumn derivatization. These derivatives showed similar intensities to aflatoxin $B_2$ and $G_2$. However, the relative instability of these derivatives and the potential advantages for automation led to the use of postcolumn derivatization techniques. Furthermore the interference that inhibits accurate quantification of aflatoxins is still noticed.

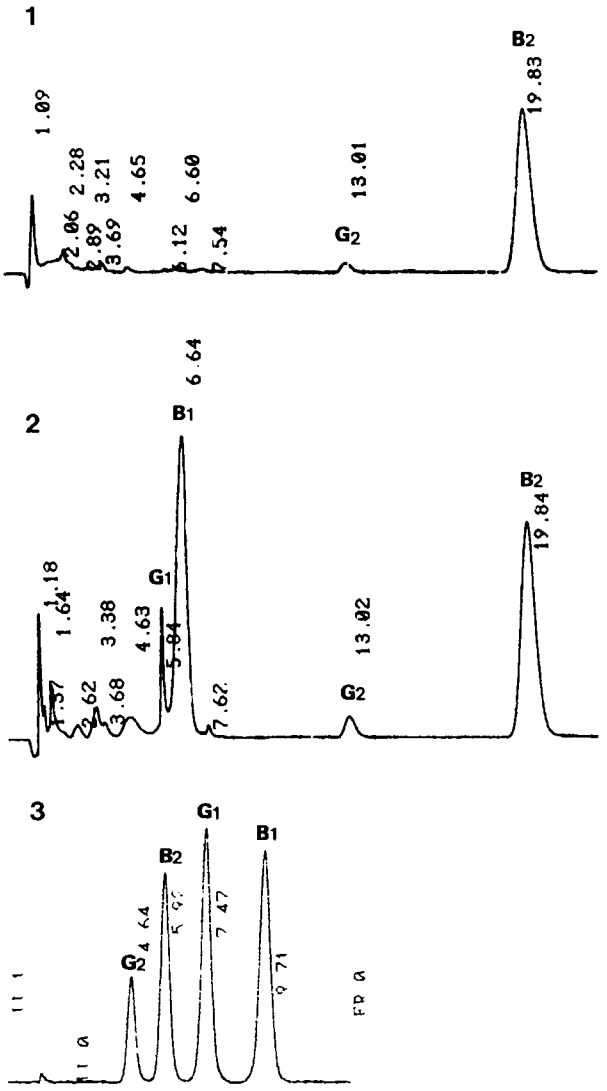


**Fig. 1. Analysis of aflatoxin standards without derivatization (1), followed by precolumn derivatization (2), and with postcolumn derivatization (3).**

Fig. 1(3) shows the results of postcolumn derivatization of aflatoxin standards. We can note how the elution order has changed so that aflatoxin $B_2$ and $G_2$ elute before aflatoxin $B_1$ and $G_1$. The four aflatoxins were clearly separated from the mixture in less than 10 minutes. The derivatization step increased the detectability of aflatoxins. This derivatization also produced almost no matrix interferences.

The four aflatoxins exhibit some degree of fluorescence, although the fluorescence varies in wavelength and intensity, and all four are also UV light absorbers, although the extinction coefficients vary. Fluorescence detection is inherently more sensitive than UV detection. The use of fluorescence detectors alone, and in conjunction with UV detectors has considerably lowered the limits of detection. Techniques such as packing flow cell with silica gel, or derivative formation to enhance detection limits have been used with success.[9-11] However, aflatoxin $B_1$ and $G_1$ do not fluorescence strongly in reverse phase solvents but strongly-fluorescing water adducts are formed readily by reacting these aflatoxins to form aflatoxin $B_{2a}$ and $G_{2a}$, so that all the four aflatoxins produce approximately equivalent fluorescence response.[13] Precolumn derivatization involves reacting the samples with trufluoracetic acid. Postcolumn derivatization is automated by an instrument with extra hardware and a saturated $Br_2$ solution.

Fig. 2 shows the linear response of the four aflatoxins in the postcolumn derivatization. In these results, the quantitative ranges of postcolumn detection were 10~100 ppb for aflatoxin $B_1$ and $G_1$ with the linearities, r=0.996 and 0.992 of calibration curves, and 3~30 ppb for $B_2$ and $G_2$ with the linearities, r=0.976 and 0.956 of calibration curves. This plot demonstrates the linearity of the converted aflatoxins, and confirms that aflatoxin $B_2$ and $G_2$ were unaffected by the derivatization procedure when compared with the previous results. Kim *et al.*[13] reported that the detection limit of the aflatoxins by the precolumn derivatization was 1 ng/injection for aflatoxin $B_1$ and $G_1$, and 0.3 ng/injection for aflatoxin $B_2$ and $G_2$. It was reported that the detection limit for aflatoxin $B_1$ was found to be about 20 pg/injection when a postcolumn reaction conditions were optimized.[14]

To find the recoveries by the postcolumn derivatization methods, an appropriate amount of mixed standard solution was added to YES broth. The spiked samples were analyzed using the derivatization methods. The recoveries were calculated as percentages as shown in Table 1. Average recoveries of aflatoxin $B_1$, $B_2$, $G_1$, and $G_2$ were 88.9%, 81.50%, 100.5%, and 87.0%, respectively. However, the postcolumn column derivatization showed relatively poor recovery for aflatoxin $B_2$ and $G_2$, and the variation was very high for these two aflatoxins. Several studies for analyses of aflatoxins in foodstuffs have shown acceptable reproducibilities, but low recoveries, the causes of which were not known.[15]

A similar procedure achieved by Trucksess *et al.*,[16] and both HPLC and the solution fluorimetry showed acceptable recoveries of above 80% for the HPLC determination and

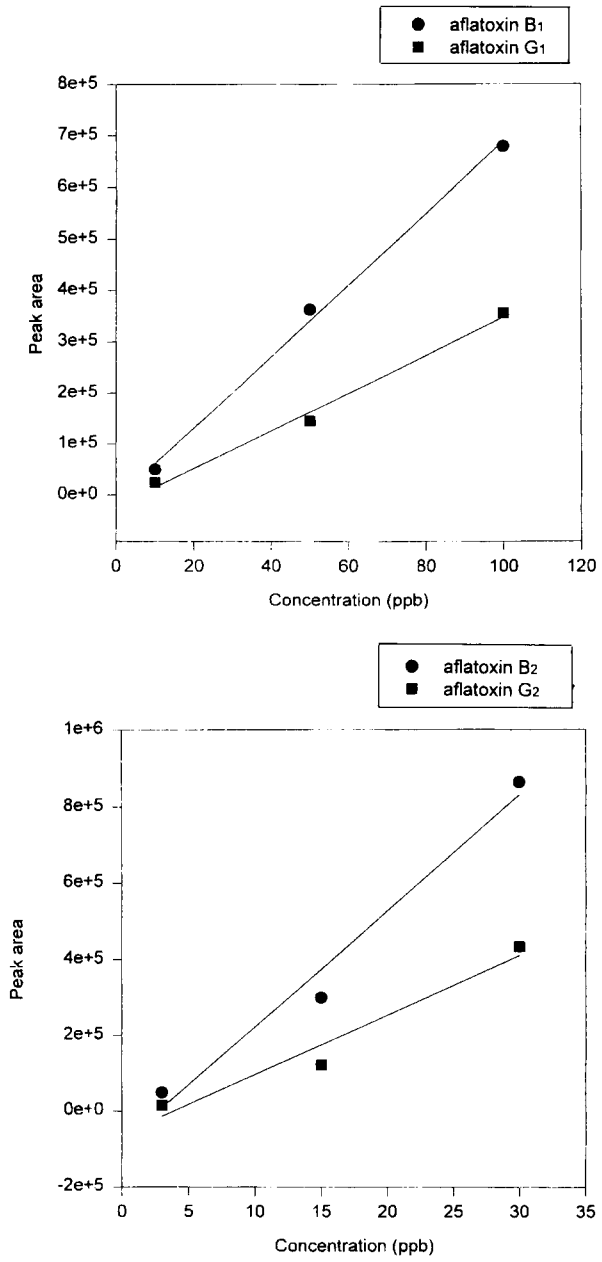


**Fig. 2. Calibration graphs for aflatoxin $B_1$ and $G_1$ using HPLC with postcolumn derivatized fluorescence detection (upper). Calibration graphs for aflatoxin $B_2$ and $G_2$ using HPLC with postcolumn derivatized fluorescence detection (lower).**

105~123% for the solution fluorimetry. Trucksess *et al.*[17] also reported that the reproducibilities at spiking levels of 10, 20, and 30 μg/kg were acceptable. The recovery percentages of aflatoxin $B_1$, $B_2$, $G_1$, and $G_2$ in the previous study using precolumn derivatization method were 82.9%, 71.5%, 80.0%, and 69.3%, respectively.[13] The postcolumn derivatization in this study was less time-consuming, safer for treatment, and proved good recovery for the two main aflatoxins ($B_1$ and $G_1$) analyzed.

**Table 1. Recovery rates of aflatoxins added to yeast-extract sucrose broth**

| Aflatoxin | Added (ppb) | Recovery (%) |
|---|---|---|
| $B_1$ | 10 | 92.2 |
| | 100 | 85.5 |
| $B_2$ | 3 | 116.6 |
| | 30 | 46.3 |
| $G_1$ | 10 | 108.3 |
| | 100 | 92.7 |
| $G_2$ | 3 | 140.3 |
| | 30 | 33.7 |

The postcolumn derivatization method was used for the determination of aflatoxins in samples (cultured media). Aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$ in the extract of cultured media were quantitatively detected by the method. Postcolumn derivatization of the aflatoxin in the samples also eliminated matrix interference problems. There was no interference that inhibited accurate quantification of aflatoxins. Precolumn derivatization of the sample converted aflatoxin $B_1$ and $G_1$ to $B_{2a}$ and $G_{2a}$, but showed unknown matrix interference problems. Based on the results, the author suggests that this postcolumn derivatization method is beneficial for the detection of aflatoxin $B_1$ and $G_1$, but further study is necessary for adequate detection of $B_2$ and $G_2$.

Since most analytes can be detected directly, precolumn and postcolumn derivatization will never be the norm for HPLC. In cases where sensitivity and selectivity pose problems, precolumn or postcolumn derivatization may be the answer. In the analysis of aflatoxins following precolumn derivatization, samples were derivatized with TFA and injected onto the reverse $C_{18}$ column. In the analysis of the toxins with postcolumn derivatization, samples were separated on a $C_{18}$ column and then derivatized on-line in a reaction system. Derivatizing aflatoxins can improve the sensitivity of the separation as Fig. 1 shows. Precolumn derivatization involved treating the sample with trifluoroacetic acid. Postcolumn derivatization was automated by an instrument with extra hardware and a saturated $Br_2$ solution. The areas and heights of the peaks representing the aflatoxins for both separations was noted. Precolumn levels

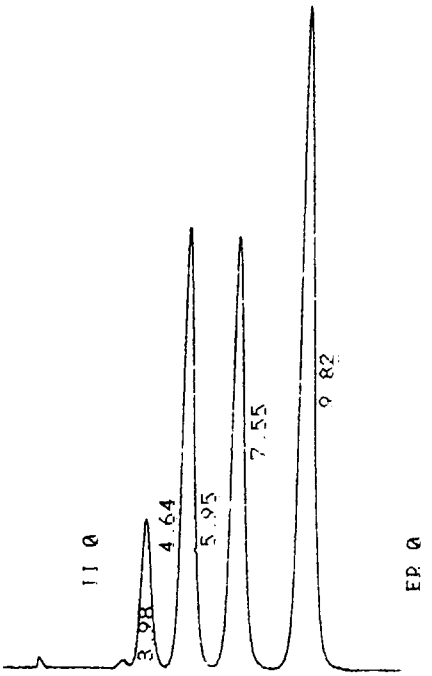


**Fig. 3. Aflatoxins recovered from yeast-extract sucreos broth.**

were lower than postcolumn levels, suggesting the postcolumn technique is approximately twice as sensitive due to the effects of an automated extra system.

**Table 2. Amount of aflatoxins in samples determined by HPLC with postcolumn derivatization**

| Sample | Aflatoxin (ppb) | | | |
|---|---|---|---|---|
| | $B_1$ | $B_2$ | $G_1$ | $G_2$ |
| 1 | 3.63 | 3.48 | 9.51 | 4.89 |
| 2 | 32.17 | 8.73 | 50.27 | 10.12 |

The advantage to precolumn derivatization is that no additional hardware is required. However, the automated postcolumn derivatization step saved time from the standpoint of sample preparation and methods development. This is just another example of how to successfully separate aflatoxins. The better sensitivity and selectivity of the postcolumn derivatization system might be expected to permit the quantification of aflatoxin residues in a variety of samples and products if aflatoxin $B_2$ and $G_2$ are also found to be detected satisfactorily.

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## 국문요약

HPLC에 의한 주요 aflatoxins(aflatoxin $B_1$, $B_2$, $G_1$ 및 $G_2$)의 동시 분석에서 postcolumn 유도체화법을 시도하였다. Electrochemical cell(Kobra-cell)을 사용한 postcolumn 유도체화법은 기존의 precolumn 유도체화법보다 분석시간을 단축하였으며(약 1/2 단축), 더 안전하고, 향상된 분석능을 보였다. Aflatoxin $B_1$과 $G_1$의 경우 10~100 ppb에서, 그리고 $B_2$와 $G_2$의 경우 3~30 ppb에서 직선성을 나타내었다. Aflatoxin $B_1$과 $G_1$은 각각 88.9% 및 100.5 %로 양호한 회수율을 보였다. Aflatoxin $B_2$와 $G_2$의 경우 분리도는 우수하였으나 회수율에 있어서 변이가 크게 나타났다.

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